

STUDIES ON VITAMIN C.

by

George T. Meiklejohn.

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INTRODUCTION.

The estimation of vitamin C in biological fluids with particular reference to urine.

The development of a satisfactory method of analysis for vitamin C is of the utmost importance from the point of view of nutritional studies, research into the biological function of the vitamin, and the detection and investigation of latent scurvy. Biological assay is tedious, expensive, and is not always applicable: chemical or biochemical methods, on the other hand, offer a much wider field of application, and, if suitably devised, are rapid, inexpensive, and accurate to a much higher degree. The work which is recounted here has been directed towards developing a biochemical method for the estimation of vitamin C which is rapid, specific and accurate.

A review of the work already carried out in this direction, especially since the elucidation of the structure and the synthesis of ascorbic acid by Herbert et al, (1933) and Reichstein et al, (1933) forms a suitable introduction to the work about to be described.

Nearly all the chemical methods so far devised for the determination of vitamin C, whether titrimetric or colorimetric, depend on the fact that ascorbic acid is a powerful

reducing agent in aqueous media. Direct methods of analysis based on the separation of the dinitrophenylhydrazine derivative of the first oxidation product of ascorbic acid, dehydroascorbic acid, have not met with general acceptance since, although this procedure is of importance in the isolation and identification of the vitamin, it cannot be carried out rapidly and quantitatively with a high degree of success.

van der Walle (1922) in an examination of urine for vitamins using the curative technique, was unable to find evidence for the presence of vitamin C. Eekelen (1933) however, found that the reducing power of urine as measured by indophenol was increased in the cases of subjects on diets including large quantities of fruit, and Harris, Ray, and Ward (1933) found that the amount of vitamin C determined by the method of Harris, Ray and Birch (1933), (*vide infra*) was increased when vitamin C was fed by the mouth. The rationale of the estimation of vitamin C in urine was not, however, established until 1937 when Meuwissens and Noyons; and Drum, Scarborough and Stewart isolated and identified ascorbic acid in normal urine. That Weiters (1935) and Ahmad (1936) had been unable to detect the vitamin is probably due to the presence of toxic substances in the urine.

Indophenol titration of ascorbic acid.

Ascorbic acid is readily oxidised in aqueous solution by iodine and other similar laboratory reagents and may be titrated thus with ease. A more specific reagent than iodine is 2:6 dichlorophenolindophenol which reacts rapidly with ascorbic acid.

Indophenol dyes have been in use for some time as redox indicators and various derivatives of indophenol have been used from time to time for the arbitrary assay of vitamin C during concentration and extraction, Zilva (1927) having used dimethylaminophenylindophenol for this purpose, while Mansfield Clark had used the 2:6-dibromophenolindophenol to demonstrate the reducing potential of some biological materials. The dye produced commercially at the present time for titration of ascorbic acid may be 2:6-dichlorophenolindophenol - sometimes called phenol-indo-2:6-dichlorophenol - or the corresponding naphthol derivative. Both, apparently, are of equal value and the samples used in this work were mostly the naphthol derivative, the molecular weight being found by direct titration to be approximately 350 and not 280 as required by the phenol derivative. The point is of some importance in the preparation of standard solutions of the dye. Standardisation of

2:6-dichlorophenolindophenol solutions, hereinafter called indophenol solutions, can be carried out by direct titration against ascorbic acid, the purity of which has been previously determined by titration against standard iodine and also sodium hydroxide. Other methods have appeared recently (Ind. Eng. Chem., Anal. Ed. 1938), some of which are based on earlier work, which make use of ferrous salts or thiosulphate solutions but the standardisation with ascorbic acid is quite convenient and accurate.

Tillmans, Hirsch et al. (1930, 1932) found that the reducing power of foodstuffs towards indophenol was parallel to the vitamin C content assayed biologically and suggested that the titration might be used to determine vitamin C in extracts. The method was further developed by Harris and Ray (1933) and also in the micro-form by Birch, Harris and Ray (1933) who stressed the necessity for carrying out the titration rapidly in acid medium, trichloroacetic acid being used for acidification. By this means, interference from sulphhydryl compounds was reduced to a minimum and the method made more specific. Bessey and King (1933) also examined this titrimetric method, pointing out that the reagent must be highly purified before use in order to obtain a clear end-point and that the solutions of the dye deteriorate fairly rapidly on

standing.

Other reducing substances present in biological material may interfere in the titration, e.g., sugar degradation products of the diénol type which react with the dye almost as rapidly as ascorbic acid itself (Euler and Martius, 1934: Birch, Harris and Ray, 1933) but the method affords a rough measure of the vitamin C present as ascorbic acid which is in good agreement with the amount assayed biologically (Lund, Spur and Fridericia, 1934: Johnstone and Zilva, 1934).

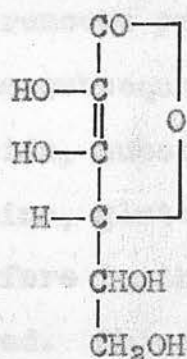
Estimation of ascorbic acid and dehydroascorbic acid.

The first oxidation product of ascorbic acid is dehydroascorbic acid with which it forms a thermodynamically reversible system. At hydrogen ion concentrations less than pH 5, the dehydroascorbic acid is unstable, and irreversible change occurring with greater ease as the hydrogen ion concentration diminishes: This change is a non-oxidative one, dependent only upon the hydrogen ion concentration and is probably associated with the opening of the lactone ring.

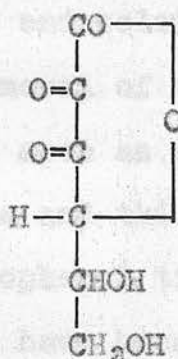
(Ball, 1937: Borsook et al., 1937)

Below pH 4, however, ascorbic acid can be recovered quantitatively from dehydroascorbic acid by reduction with hydrogen sulphide or sulphydryl

compounds such as cysteine or glutathione.



Ascorbic acid.



Dehydroascorbic acid.



2:3-diketo-gulonic acid.

Ascorbic acid is also regenerated by nascent hydrogen. The reduction by means of hydrogen sulphide to ascorbic acid after oxidation by iodine or hydrogen peroxide was mentioned by Tillmans (1932) but was not incorporated as part of an analytical procedure until 1933 when Emmerie and van Eekelen showed that the indophenol titre of urine increased after a preliminary reduction with hydrogen sulphide. Since both ascorbic acid and dehydroascorbic acid are active physiologically to the same extent, (Demole, 1933; Zilva, 1928; Fox and Levy, 1936; Borsook et al., 1937) it is of the utmost importance to estimate not only ascorbic acid but dehydroascorbic acid as well in an analysis for vitamin C.

A preliminary reduction with hydrogen sulphide was included in the analytical procedure of Emmerie and van Eekelen (1934) who also introduced a mercuric acetate or barium acetate precipitation.

This precipitation, in the case of urine, not only removes protein and colouring material but, in the subsequent removal of the mercury as the sulphide, substances such as cysteine, ergothionine, glutathione and thiosulphate which interfere in the indophenol titration are also removed. Objections have been raised to the mercuric acetate precipitation by Evelyn, Malloy and Rosen (1938) who prefer the barium method, but other authors have confirmed the utility of mercuric acetate in removing interfering substances without affecting the ascorbic acid present.

(Stewart and Scarborough, 1937). Degradation products of sugars and other dienol compounds which react more or less rapidly with indophenol are still included in the titration after the procedure of van Eekelen; the method is not specific.

Estimation of true vitamin C by enzymatic methods.

The highly specific nature of many enzymatic reactions affords an excellent method of conducting an analysis for the substrate of the reaction even in the presence of chemically similar substances which might make it difficult and even impossible to carry out a strictly chemical analysis with the same degree of specificity. A fair number of enzymatic determinations are now in general use,

e.g., urease for urea, the dehydrogenases of lactic, malic and succinic acids for their respective substrates, choline esterase for acetylcholine, special yeast strains for the determination of sugars such as galactose.

The discovery by Sz. Györgi in 1928 of an enzyme which oxidised ascorbic acid rapidly led to the suggestion that the enzyme might be specific and play a part in the biological function of vitamin C. It also provided a method of determination of ascorbic in the presence of other reducing substances and various analytical procedures for the specific determination of ascorbic acid were suggested by Tauber and Kleiner (1935), Srinivasan (1937) and Scarborough and Stewart (1937), although at that time the enzymatic nature of the reaction had been questioned by Barron, Barron and Klemperer (1936) and Stotz, Harrer and King (1937), and there was no evidence at all for the specificity of the enzyme.

The procedure adopted in the enzymatic determination of ascorbic acid is as follows: after a preliminary treatment and reduction based on the method of Emmerie and van Eekelen, the indophenol reducing power of the final filtrate is measured after an aliquot has been taken for enzymatic oxidation. Oxidation is carried out by buffering the acid solution with a concentrated solution of sodium hydroxide until the pH is 6.0

and aerating for some time. After complete oxidation with air, which occurs within 1/2 hour, the solution is made strongly acid by the addition of m-phosphoric acid, the protein precipitate centrifuged off, and the supernatant liquid titrated with indophenol. The difference between the two titres, due allowance having been made for the dilutions involved, determines the amount of true ascorbic acid in as far as the enzyme is specific. All three groups of workers who used the enzymatic procedure found that there was present in biological materials an indophenol reducing substance, in larger quantities than the equivalent of the blank, which was not oxidised by the enzyme. The results yielded by this procedure are, so far, the most accurate obtainable. There is, of course, a certain amount of error involved in the unavoidable loss of dehydroascorbic acid in biological materials where the pH is greater than 5.0 and there is no protective mechanism to prevent this disappearance.

(Barron, Barron and Klemperer, 1936: Borsook et al., 1937).

Other methods of estimating vitamin C.

From time to time other methods for the determination of vitamin C have been proposed: most of these depend for their function on the reducing power of ascorbic acid and, hence, are subject to the same sources of interference as the indophenol titration method outlined above. Medes (1933) bases a colorimetric method on the reduction of phospho-18-tungstic acid, and Gal (1936) proposed the use of an excess of methylene blue followed by a back-titration with titanium trichloride. As they stand, these methods are, if anything, less specific than the indophenol method. Roe (1936) suggested the conversion of ascorbic acid to furfural and subsequent determination of the furfural colorimetrically with aniline acetate. The extract for analysis by this method is cleared by norite, thus reversibly oxidising the ascorbic acid, and the difference in yield of furfural when the conversion is carried out in 30% hydrochloric acid plus 10% stannous chloride, and in 30% hydrochloric acid alone, measures the amount of the vitamin. The method has been developed further by Roe and Hall (1939), the dehydroascorbic acid being separated as the dinitrophenylhydrazine derivative and the subsequent determination being carried out as before. The conditions appear to be somewhat

drastic for reliable analyses.

Bessey (1937) and Evelyn, Malloy and Rosen (1937) have both developed photoelectric colorimetric methods based on the decolourisation of indophenol using extrapolation of the dye-reduction-time curve to correct for the influence of interfering substances. The method, however, offers no great advantage over the titrimetric procedure.

Kassel and Brand (1938) have modified the Folin-Lugg procedure for the analysis of urine with a high sulphur content so that cystine, cysteine, and ascorbic acid can be measured colorimetrically. The method and analysis of results are rather complicated. Rosenthaler (1938) proposed the use of cacothelin, a reagent normally used for the detection of stannous ions, but some experiments carried out two years ago by the present author gave no indication that the reagent could be used with advantage for the determination of ascorbic acid. The reduction of an azo dye is the basis of a method proposed by Scudi and Ratish (1938) but, as with other similar methods, interference occurs, principally from hydroquinones and glucoreductones. Espil and Genevois (1939) separate the vitamin as the osazone of dehydroascorbic acid and titrate this product with titanium trichloride. Incidentally,

it may be stated here that the rationale of a method using dehydroascorbic acid as its basis rather than ascorbic acid is a good one provided that conditions do not permit the disappearance of dehydroascorbic acid since the oxidative reaction is so much more easy to perform than the reverse reaction. In urine, however, the isolation of the osazone of dehydroascorbic acid is by no means a specific process. (Scarborough and Stewart, 1936).

Fujita and Sakamoto (1939) have estimated ascorbic acid by a direct spectroscopic method, the absorption maximum at 2400 \AA being used. against iodine and also sodium hydroxide, and was found to be $110,000$ by the former and $90,100$ by the latter. Indophenol solutions were prepared from powdered or crystalline 2,6-dichlorophenolindophenol and were standardized against amounts of ascorbic acid weighed out in a microbalance. Three stock solutions were used: 1 ml being equivalent to 0.01 mg , 0.05 mg , or 0.1 mg ascorbic acid. The most dilute solution was occasionally prepared for urine titration from the standard tablets of the dye manufactured commercially which are equivalent to 1 mg ascorbic acid, and are accurate to within 1% . The solutions were stored in the ice chest and were retained only three days.

EXPERIMENTAL PROCEDURE.

Glass distilled water was used throughout, and the reagents were of "Analar" or A.R. standard of purity. Glacial acetic acid was distilled once from dichromate to minimise the tendency to de-colourise indophenol when in contact with the dye for some time. Hydrogen sulphide was generated in a large Kipp apparatus and carbon dioxide and nitrogen were obtained from cylinders of the gases: all three were thoroughly washed in gas wash bottles before use. The ascorbic acid used, (synthetic, Hoffman la Roche) was standardised against iodine and also sodium hydroxide, and was found to be 100,00% by the former and 99,10% by the latter. Indophenol solutions were prepared from powdered or crystalline 2:6-dichlorophenolindophenol and were standardised against amounts of ascorbic acid weighed out in a microbalance. Three stock solutions were used: 1 ml being equivalent to 0,01 mg, 0,05 mg, or 0,10 mg ascorbic acid: the most dilute solution was occasionally prepared for urine titration from the standard tablets of the dye manufactured commercially which are equivalent to 1 mg ascorbic acid, and are accurate to within 1%. The solutions were stored in the ice chest and were retained only three days.

Urine.

The urine used was freshly passed or a 24 hour specimen according to the experiment, and, unless otherwise stated, was normal. Acetic acid was added to each specimen to give a final concentration of 10%. The problem of the disappearance of dehydroascorbic acid in 24 hour specimens was overcome by the addition of 1/20th volume of the stannous chloride solution described below. This prevented the irreversible oxidation of ascorbic acid and in no way interfered with the subsequent procedure.

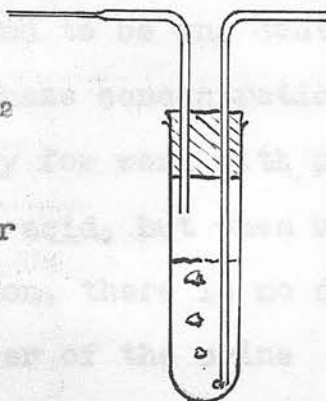
Clearing.

Two routine methods of clearing were used. The first is that of Scarborough and Stewart (1937) for "clearing" urine.

20 mls urine + 20 mls 20% mercuric acetate in 10% acetic acid + 20 mls 10% acetic acid are mixed, centrifuged quickly (three minutes) and hydrogen sulphide passed immediately through the decanted supernatant liquid. The black precipitate is filtered off and

made free of H_2S by bubbling through it a stream of wet CO_2 or N_2 in the apparatus shown in Fig. 1. Lead acetate paper was used to test the effluent gas for hydrogen sulphide and

Figure 1.



blank experiments showed that this was sufficiently sensitive.

The second clearing method is used after reduction of dehydroascorbic acid solutions with stannous chloride when these happen to be coloured; in the case of clear solutions the tin is precipitated directly as the sulphide. A measured and usually small volume of 20% mercuric acetate in 10% acetic acid is added to the solution containing stannous chloride etc., and the whole quickly saturated with hydrogen sulphide. The precipitated sulphides are filtered off and the filtrate aerated as before to remove H_2S (10 mins.).

Stannous chloride.

Stannous chloride is stable only in strongly acid solution and some difficulty was experienced in the preparation of suitably dilute solutions which contained a low enough concentration of chloride ions, these having been shown to accelerate the decomposition of ascorbic acid (Lyman, Schultze and King, 1937). By a process of trial and error, the most suitable solution for the reduction of dehydroascorbic acid was found to be one containing $M/10$ $SnCl_2$ in $M/4$ HCl : these concentrations are higher than are necessary for work with pure solutions of dehydroascorbic acid, but when urines are treated with this solution, there is no danger of the natural buffering power of the urine

bringing the hydrogen ion concentration into the range in which the tin is precipitated as the oxychloride or a stannate. The solution is prepared by dissolving 22.25 gms $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ in 25 mls concentrated hydrochloric acid with heat and diluting to 1 litre: the solution keeps well for a month or two on the addition of a very small piece of granulated tin. 5 mls of this solution are added to the solution or filtrate for reduction - containing not more than 100 mg dehydroascorbic acid - and the mixture allowed to stand for 10 to 15 minutes before removal of the tin. The stannous chloride solution issued by B.D.H. Ltd. for arsenic testing is also very useful as a stock solution of stannous chloride, 0.20 ml being added to the solution or filtrate containing not more than 100 mg dehydroascorbic acid. This solution was employed mostly in the micro-method discussed below since the volume increase was very low and great dilution could be avoided.

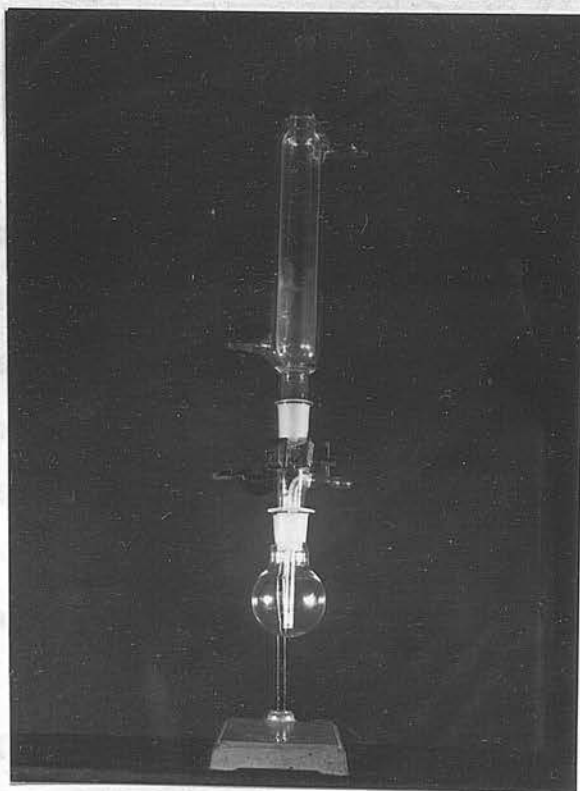
Reduction by hydrogen sulphide.

The solution or filtrate for reduction - usually 20 mls - was saturated by bubbling H_2S through for 30 minutes: the test tube was then stoppered tightly and allowed to stand for 24 hours before blowing off the H_2S as described above (30 minutes).

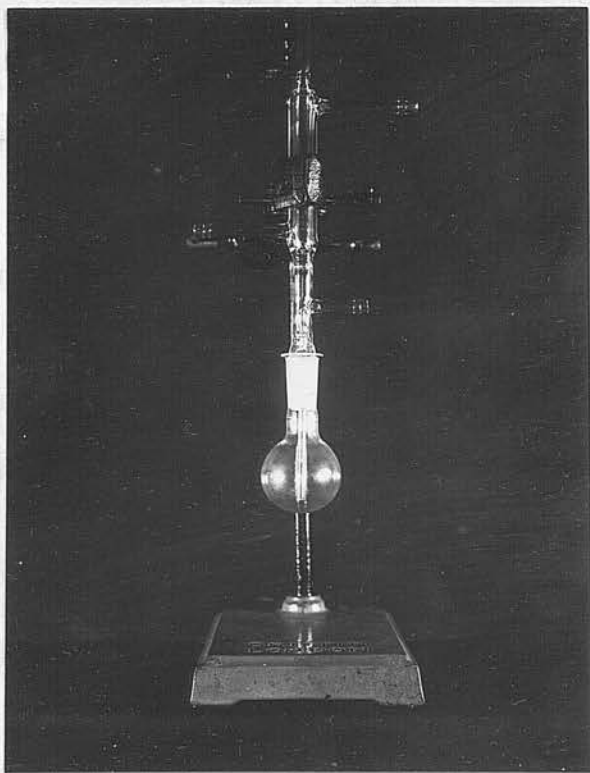
Hydrolysis.

Hydrolysis was carried out by refluxing in acid solution (urine filtrates being themselves acid) for a measured interval of time in the reflux apparatus illustrated in Fig. 2. 50 mls to 100 mls were used in the macro-apparatus and 20 mls or 25 mls were used in the micro-apparatus. Correction to the volume was applied every time to allow for any slight loss of fluid.

Figure 2.



Macro-apparatus



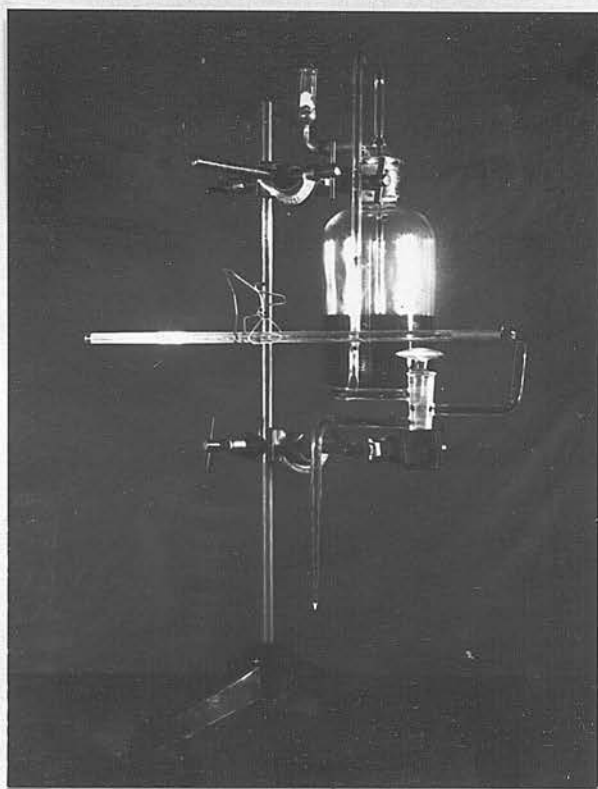
Micro-apparatus.

Titration.

Indophenol titrations were carried out as quickly as possible in acid solution, glacial acetic acid or m-phosphoric acid being used to acidify solutions previously buffered; the end-point was the non-reappearance of a perceptible pink colour after 10 seconds when the reducing filtrate was run from the burette into a measured volume of indophenol solution, or the persistence of a perceptible pink after 10 seconds when the indophenol solution was run in from a burette. After some experience with the dyestuff, accurate endpoints were obtained by both methods which

agreed very closely or exactly. Comparison tubes were used if the filtrate was slightly coloured. The burettes used were 5 or 10 ml semi-micro-burettes, graduated in 1/100th millilitre, and the titrations were carried out in small Pyrex conical flasks or Pyrex centrifuge tubes. A modification of the Conway burette (Micro-diffusion analysis and Volumetric Error, Conway: London, 1939) which was developed in the Biochemical Laboratory, Cambridge, the design of which was kindly supplied by Dr. Elsdon, was used for the micro-titrations and is illustrated in Fig. 3.

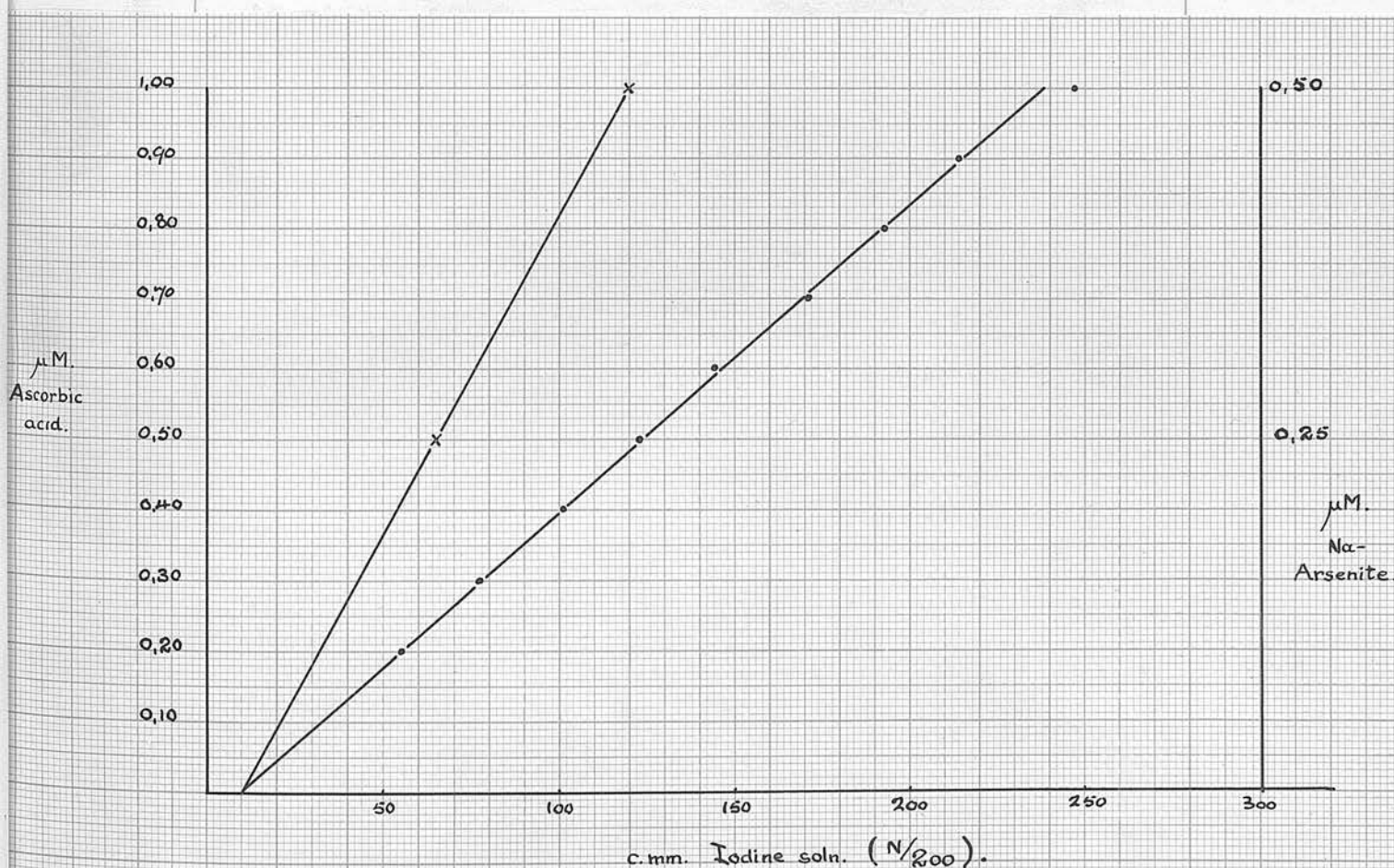
Figure 3.



Micro diffusion-burette.

The titration in this case is a diffusion one and a capillary air stirrer was fitted, the standard volume of solution for titration being 2,0 mls and the capacity of the burette 250 c.mm. N/200 iodine was used in dealing with pure solutions because of the sharper endpoint obtainable, and indophenol was used for all other titrations. A calibration curve for iodine-ascorbic acid and standardisation of iodine by arsenite is shown in Fig. 4.

Figure 4.



Titration Blank : 10 c.mm. (from graph).

9.5 - 11.0 c.mm. (by experiment).

Enzyme oxidation.

The acid filtrates obtained after reduction and hydrolysis were buffered with 25% sodium hydroxide to pH 6,0 using Brom-cresol purple (5,2 - 6,8) as an external indicator, the volume of sodium hydroxide being measured from a burette. The enzyme solution was prepared by freezing a cucumber overnight and allowing a section to autolyse at room temperature. The expressed juice of a freshly thawed portion was tested for activity and experience showed that a fresh and active sample of juice remained active for several days if stored in the ice chest. The juice was used as the enzyme solution without further treatment. 1 ml of enzyme solution was added to the buffered filtrate (approximately 20 - 25 mls) and the whole aerated for 30 minutes in the Blood Urea apparatus manufactured by Quickfit and Quartz, Ltd., after which time the aeration was stopped and the enzyme activity brought to an end by the addition of 2 mls 50% m-phosphoric acid. The solution was titrated after the protein precipitate had been removed by centrifugation.

Preparation of reductic acid and gluco-reductone.

Reductic acid was prepared according to the method of Reichstein et al., *Helv. Chim. Acta.*, 1933, 16 988. Two samples were prepared, one by autoclaving citrus pectin directly, and the other by preparing first tetragalacturonic acid from pectin by 6 hours' hydrolysis in 5% sulphuric acid and removal of the sludge of the galacturonic

acid by means of a Sharpel centrifuge. About 500 mg of reductic acid were obtained in all. m.p. 210°C (uncorrected). Quoted m.p. 213°C .

Reductone was prepared by the method of Euler and Martius, Ark. Kemi. Min. Geol., 11B, Nos. 8 and 12; 1933. The material was not isolated but solutions were prepared freshly before use.

The introduction in the estimation of vitamin C in urine and provides an accurate means of assay. It is, however, prolonged, and suffers the disadvantage that the final titrations are carried out in filtrates which are several times more dilute than the original urine, thus making the end-point of the indophenol titration a matter of some difficulty. An examination of the method was undertaken since indications had presented themselves that part of the method required revision, and also to attempt to reduce the time required for a single estimation.

The prolonged hydrolysis by refluxing the urine for 24 hours was investigated.

Samples of urine were cleared, and reduced with hydrogen sulphide, ascorbic acid in weighed amounts being added to aliquots of each sample to study the effect of the hydrolysis on higher concentrations of ascorbic acid. Samples were removed at fixed intervals and titrated against indophenol after reduction and clearing; the results are expressed graphically in Figure 5.

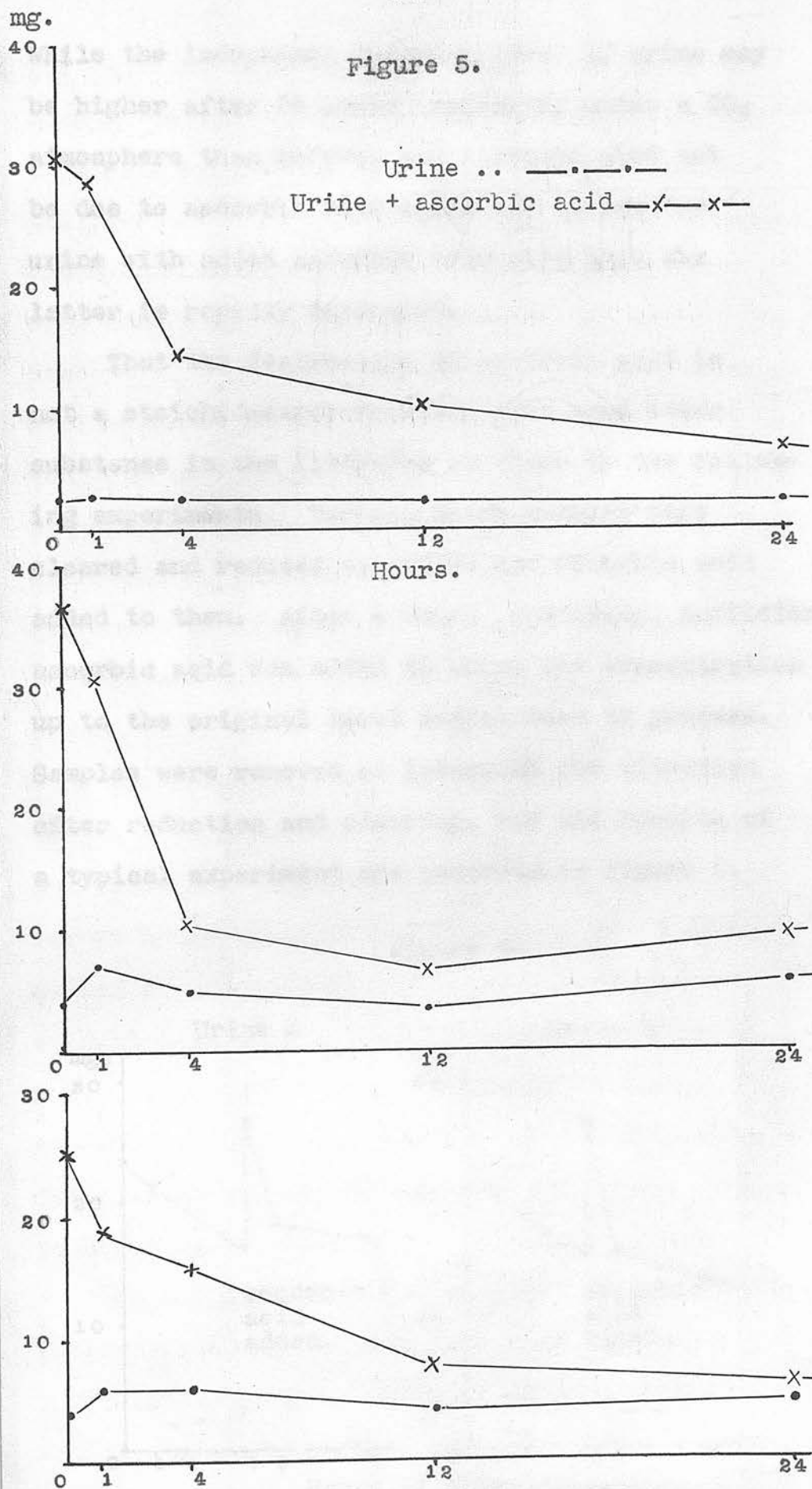
DISCUSSION and RESULTS.

The method of Scarborough and Stewart (1937) overcame most of the difficulties mentioned in the introduction in the estimation of vitaminC in urine and provides an accurate means of assay. It is, however, prolonged, and suffers the disadvantage that the final titrations are carried out in filtrates which are several times more dilute than the original urine, thus making the endpoint of the indophenol titration a matter of some difficulty. An examination of the method was undertaken since indications had presented themselves that part of the method required revision, and also to attempt to reduce the time required for a single estimation.

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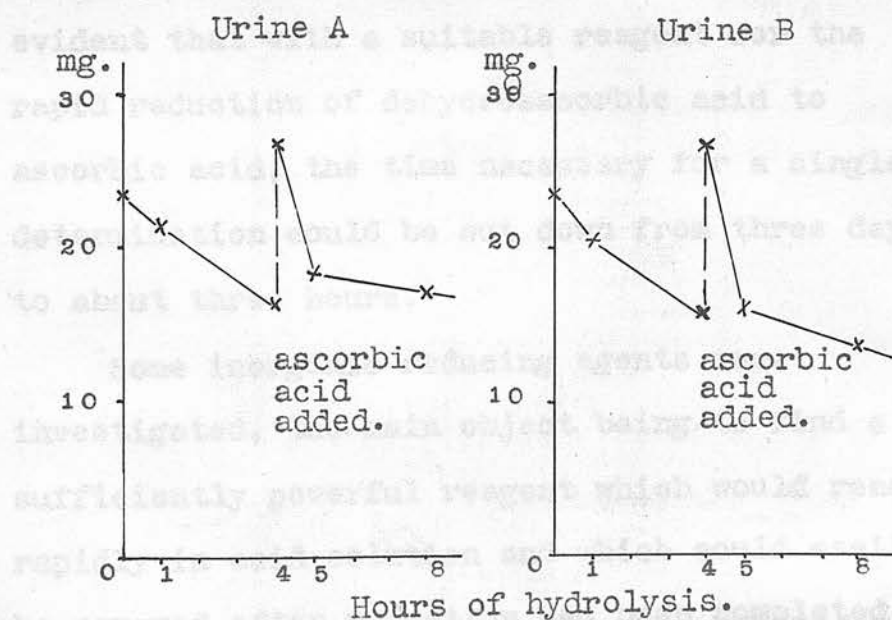
Figure 5.



While the indophenol reducing power of urine may be higher after 24 hours' refluxing under a CO_2 atmosphere than before, the increase need not be due to ascorbic acid since the curves for urine with added ascorbic acid show that the latter is rapidly destroyed.

That the destruction of ascorbic acid is not a stoichiometric reaction with some other substance in the filtrates is shown by the following experiments. Various urine samples were cleared and reduced as before and ascorbic acid added to them. After 4 hours' refluxing, sufficient ascorbic acid was added to bring the concentration up to the original level and ^{the refluxing} allowed to proceed. Samples were removed at intervals for titration after reduction and clearing, and the results of a typical experiment are recorded in Figure 6.

Figure 6.



The rapid rate of disappearance of the ascorbic acid which was added after refluxing for 4 hours - at a rate comparable to the disappearance during the first hour of hydrolysis - suggests a catalytic decomposition rather than a stoichiometric reaction with some other substance. The amount of copper present in a urine filtrate even after the precipitation of mercury with hydrogen sulphide would be sufficient to account for the disappearance but the anaerobic conditions prevailing make it difficult to explain the source, apparently difficult to exhaust, of the oxidising agent if such is responsible for the decrease in reducing power.

The graphs of Fig. 5 give some indication that the main part of the hydrolysis occurs in the first hour and that the prolonged hydrolysis for 24 hours could be excluded from the general method of Scarborough and Stewart. It was also evident that with a suitable reagent for the rapid reduction of dehydroascorbic acid to ascorbic acid, the time necessary for a single determination could be cut down from three days to about three hours.

Some inorganic reducing agents were investigated, the main object being to find a sufficiently powerful reagent which would react rapidly in acid solution and which could easily be removed after reduction had been completed.

Preliminary experiments with titanous chloride solution were not encouraging, the recovery of ascorbic acid from dehydroascorbic acid being negligible, but stannous chloride in fairly dilute hydrochloric acid solution reduced dehydroascorbic acid quite rapidly and could be removed by a very quick precipitation of stannous sulphide, or by clearing with mercuric acetate in the normal manner.

The preliminary experiments in which dehydro-ascorbic acid, prepared by treating a standard solution of ascorbic acid with charcoal, was treated with a large excess of stannous chloride in dilute hydrochloric acid solution for 20 - 30 minutes with subsequent removal of the tin as sulphide, gave the following results:

Table I.

Figures represent mg ascorbic acid in 20 mls soln.
(corrections applied for dilution)

Ascorbic acid solutions.		Recovered Ascorbic acid.	Recovery.
Before oxidation.	After oxidation.		
0,39	0,01	0,36	92%
3,20	0,02	3,12	98%
3,32	0,02	3,02	91%
6,64	0,01	6,36	96%

The results were sufficiently encouraging to justify an attempt to standardise the conditions of reduction by stannous chloride and removal of the tin in order to obtain a quantitative recovery

of ascorbic acid from dehydroascorbic acid. The stannous chloride solution described in Experimental Procedure was found to reduce dehydroascorbic acid quantitatively in pure solution and in urine and Table II shows recoveries obtained in 5 ml portions of dehydroascorbic acid solutions, the micro-diffusion burette being used for the titrations.

Table II.

Figures represent mg ascorbic acid in 5 mls soln.
(corrections applied for dilution)

Ascorbic acid solutions.

Before oxidation.	After oxidation.	Recovered ascorbic acid.	Recovery.
0,050	0,000	0,050	100%
0,086	0,001	0,087	101%
0,102	0,000	0,100	98%
0,490	0,005	0,491	100%
0,671	0,001	0,670	100%
0,880	0,002	0,878	99%

When the above method of reduction was applied to urine on the macro scale and the increase in indophenol reducing power compared with that obtained by reduction with hydrogen sulphide for 24 hours, the results were not strictly parallel, a fact which is due to the difference between the hydrolytic actions during 10 minutes in contact with stannous chloride solution and saturation with hydrogen sulphide for 24 hours. The hydrolysis and reduction were therefore combined in one operation after the following experiments had

shown that ascorbic acid was quantitatively recoverable after refluxing for 4 hours on the hot water bath in presence of stannous chloride solution and under an atmosphere of nitrogen. The results in which various reagents were used for acidification are expressed in Table III.

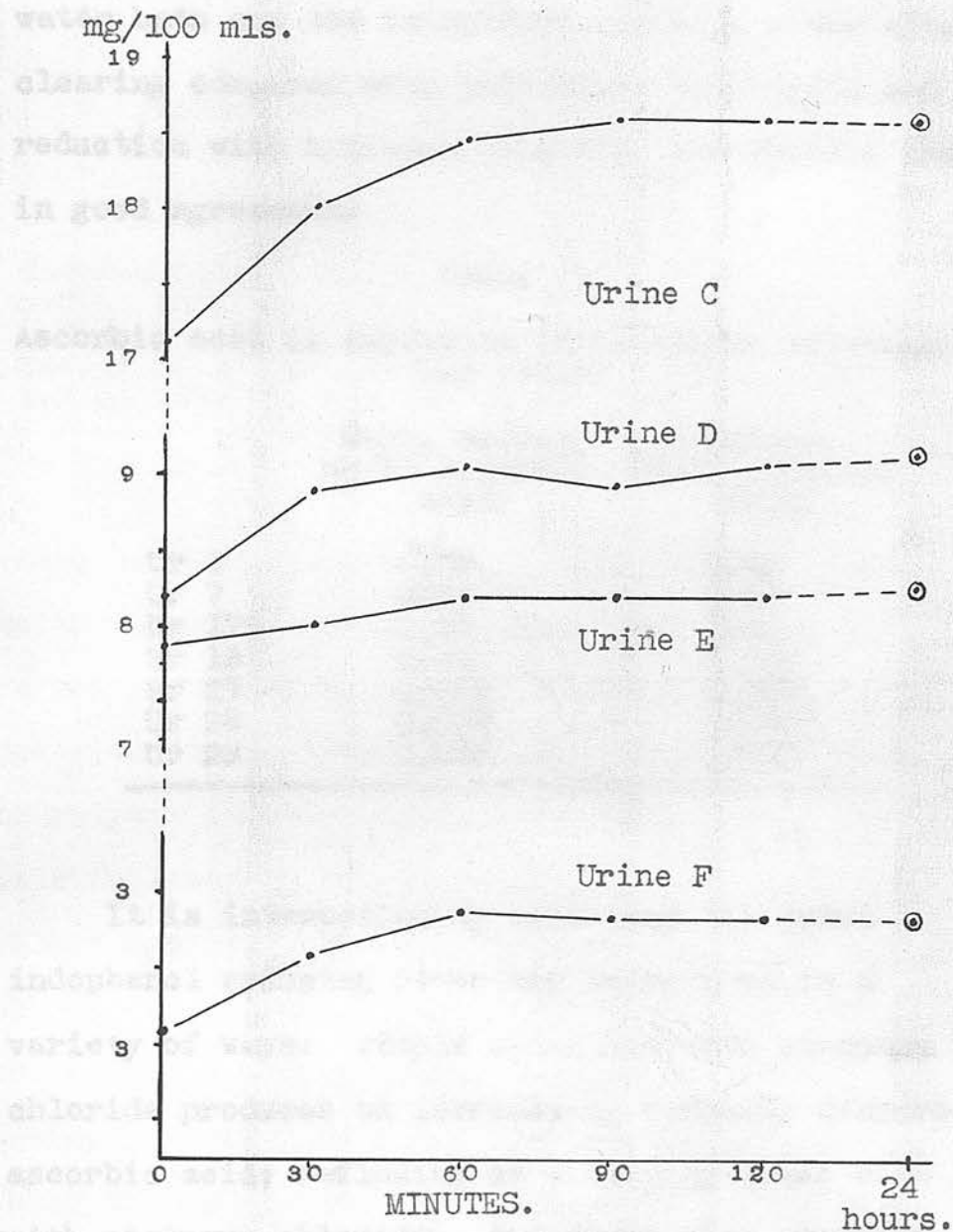
Table III.

mg ascorbic acid.	Solvent.	ascorbic acid (mg) recovered.	Recovery.
12,3	N/100 HCl	11,9	97%
10,5	do.	10,2	97%
8,23	5% HAc.	8,22	100%
21,0	do.	21,6	103%
5,50	1,5% HPO ₃	5,50	100%
4,27	do.	4,27	100%
21,0	10% HAc.	20,5	98%
12,5	do.	12,5	100%

The application of the method to urine was investigated to determine firstly, the time necessary for total reduction and hydrolysis in presence of stannous chloride solution and secondly, whether this procedure yielded similar results to the method of Scarborough and Stewart which had been modified by the exclusion of the prolonged period of refluxing. Urine samples were therefore submitted to hydrolysis in presence of 1/5 volumes of stannous chloride solution and samples were removed at intervals for titration after clearing. Aliquots of the same urines were hydrolysed and reduced by prolonged treatment

with hydrogen sulphide, and the results are expressed graphically in Figure 7.

Figure 7.



The indophenol reducing power apparently reaches a maximum after some 60 to 90 minutes and this value is the same as that obtained by treatment

with hydrogen sulphide: the 90 minute interval was taken as a standard time for routine purposes. Some comparative results are tabulated in Table IV below, the urine samples were treated with stannous chloride solution for 90 minutes on a boiling water bath and the indophenol reducing power after clearing compared with that after hydrolysis and reduction with hydrogen sulphide. The results are in good agreement.

Table IV.

Ascorbic acid is expressed as milligram molecules per litre.

	SnCl ₂ method. mm/l. ascorbic acid.	H ₂ S method. mm/l. ascorbic acid.
Ur 6	0,43	0,42
Ur 7	0,37	0,36
Ur 17	0,30	0,30
Ur 19	0,210	0,205
Ur 27	0,059	0,059
Ur 28	0,074	0,077
Ur 29	0,055	0,053

It is interesting to note that the total indophenol reducing power may be reached in a variety of ways: simple reduction with stannous chloride produces an increase by reducing dehydro-ascorbic acid; refluxing on a boiling-water bath with stannous chloride, treatment with stannous chloride in the cold for 24 hours, and hydrolysis and reduction by hydrogen sulphide all produce a greater and constant increase in the indophenol reducing power determined without such treatment.

In Table V below are collected the indophenol reducing powers of several urine samples determined by the various methods mentioned above, all filtrates being cleared immediately before titration.

Table V.

Figures represent mm/l. ascorbic acid.

	Ur 21.	Ur 19.	Ur 17.	Ur 10.
Cleared only.	0,419	0,241	0,188	0,054
SnCl ₂ : 10 mins.	0,492	0,316	0,218	0,055
SnCl ₂ : 90 mins.	0,550	0,295	0,259	0,146
SnCl ₂ : 24 hours	0,548	0,298	0,259	0,145
H ₂ S method.	0.551	0,296	0,257	0,146

There is good concordance between the different methods, and it is evident that simple reduction is not enough to increase the indophenol reducing power to its maximum. On the other hand, simple hydrolysis in acid solution for 24 hours in the cold followed by simple reduction with stannous chloride rarely produced the same result as any of the above methods, a fact which indicates that the hydrolysis only proceeds regularly when accompanied by reduction: the irregularities found in the 24 hour period of refluxing in the method of Scarborough and Stewart might have been avoided if reduction had taken place simultaneously.

The suggestion made by Scarborough and

Stewart that ascorbic acid is present in urine partly in a non-reducing combined form is borne out by the above and other results. Short treatment with stannous chloride in the cold (see Table V) increases the indophenol reducing power of urine to a definite extent, viz., the amount of dehydroascorbic acid present. Prolonged treatment with stannous chloride or treatment at 90°C for 90 minutes both increase the indophenol reducing power to a greater and equal extent: the reaction in this case is not one of simple reduction over a longer period of time since 10 - 15 minutes is a sufficient length of time for complete reduction of dehydroascorbic acid by stannous chloride. Unless there is some mechanism whereby the simple reduction of dehydroascorbic acid is considerably slowed down, the only explanation is that the prolonged treatment in the cold or the shorter treatment at higher temperatures is a hydrolysis liberating a substance which, under the reducing potential, becomes itself a reducing agent. Experiments with enzymes discussed below indicate that the extra reducing power after hydrolysis is due to ascorbic acid.

As a final and necessary test of the stannous chloride method, the "true" ascorbic acid as measured by the enzymatic method was determined in filtrates treated by both the stannous chloride and hydrogen sulphide methods. The

results are given in Table VI.

Table VI.

The urines examined here are from a scorbutic subject before and after treatment with vitamin C.

Figures represent reducing powers expressed as mM/l. ascorbic acid. x = non-vitamin material.

	Before oxidation.		After. (x).		Difference. (ascorbic acid)	
	SnCl ₂ .	H ₂ S.	SnCl ₂ .	H ₂ S.	SnCl ₂ .	H ₂ S.
Ur 1 ₁	0,149	0,147	0,060	0,059	0,089	0,088
Ur 4 ₁	0,157	0,152	0,077	0,071	0,080	0,081
Ur 11 ₁	0,041	0,039	0,043	0,040	—	—
Ur 16 ₁	0,053	0,052	0,035	0,034	0,018	0,016
Ur 15 ₂	0,904	0,898	0,040	0,040	0,864	0,858
Ur 16 ₂	0,381	0,378	0,045	0,042	0,336	0,336
Ur 17 ₂	0,925	0,925	0,067	0,069	0,858	0,856

The agreement between the two methods is satisfactory.

Specificity of the enzyme under the conditions of analysis.

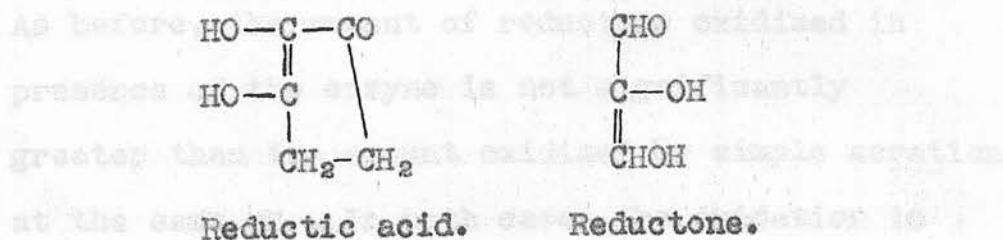
The specificity of the method depends to a great extent upon the substrate specificity of the enzyme. At the time of inception of the enzymatic determination of ascorbic acid, there was no evidence whatever for the substrate specificity but in 1938, Zilva and Snow provided evidence that the enzyme was not specific but oxidised many other related compounds which had as part of their structure the dienol group which is the main functional group of ascorbic acid.

Nevertheless, it seemed that the inclusion of

the enzymatic procedure in the analytical method was still desirable since, UNDER THE CONDITIONS OF ANALYSIS, ascorbic acid is oxidised completely whereas two other dienol compounds examined are only oxidised to the extent of some 50%. Furthermore, there is as yet no evidence that these other substances are present in normal or scorbutic urines. The rates of oxidation by the enzyme of reductic acid and reductone, the two compounds examined, are not significantly greater than the rates of oxidation by simple aeration at pH 6,0 in absence of the enzyme: under the conditions of analysis there is bound to be a fair amount of catalytic oxidation by heavy metals.

Thus, although it cannot be claimed that the method of analysis is completely and absolutely specific, interference is limited to a few compounds, of the same dienol type as ascorbic acid, whose presence in urine is not yet proved.

The experiments on the specificity were carried out in exactly the same manner as the enzymatic oxidations in the analytical procedure, using reductic acid and reductone as substrates.



Control tubes containing known amounts of ascorbic acid of the same molecular concentration

were set up each time and the ascorbic acid was completely oxidised. The pH was checked before and after aeration in the Beckman pH meter.

Table VII.

Figures represent total reductic acid in solution.

Reductic acid solution.	Aerated at pH 6,0 + enzyme.	Aerated at pH 6,0.
0,270 mM	0,160 mM	0,179 mM
0,164 mM	0,083 mM	0,083 mM
0,083 mM	0,025 mM	0,024 mM

The oxidation in 30 minutes is quite incomplete and is not significantly greater in presence of the enzyme than in its absence.

Table VIII.

Figures represent total reductone in solution.

Reductone solution.	Aerated at pH 6,0 + enzyme.	Aerated at pH 6,0.
0,375 mM	0,148 mM	0,182 mM
0,624 mM	0,431 mM	0,432 mM
0,740 mM	0,446 mM	0,456 mM
0,787 mM	0,313 mM	0,388 mM
0,948 mM	0,406 mM	0,415 mM
1,301 mM	0,662 mM	0,762 mM

As before, the amount of reductone oxidised in presence of the enzyme is not significantly greater than the amount oxidised by simple aeration at the same pH. In both cases the oxidation in 30 minutes is incomplete.

It is suggested that the utility of the enzymatic procedure for the determination of vitamin C is upheld by these results.

If it is assumed that the non-vitamin material which reduces indophenol after enzymatic oxidation is one or several of the dienol compounds similar to or identical with reductone and reductic acid, results of the highest accuracy can be obtained by subtracting from the total indophenol reducing power twice the ascorbic acid equivalent of the non-enzyme-oxidisable residue since only 50% of the non-vitamin reducing material disappears during the course of enzyme oxidation.

Routine procedure for the determination of vitamin C in urine.

Reagents: Glacial acetic acid and 10% acetic acid.
 20% mercuric acetate in 10% acetic acid.
 M/10 stannous chloride in M/4 hydrochloric acid solution, or B.D.H. solution for arsenic testing.
 H_2S gas, and CO_2 and/or N_2 .
 Indophenol solution: 10 mg per 100 mls.
 25% sodium hydroxide.
 50% m-phosphoric acid, freshly prepared.
 Autolysed cucumber juice.

Procedure, macro-method:

The urine need not be cleared beforehand unless it has a high specific gravity, is darkly coloured, opaque, and likely to contain large amounts of interfering substances as in the case

of high sulphur urines.

To 50 mls of the urine or urine filtrate after clearing, 10 mls stannous chloride solution (or 0,50 ml B.D.H. reagent) are added and the whole refluxed under an inert atmosphere, preferably nitrogen, for 90 minutes. Cool quickly, add 20 mls mercuric acetate solution, pass hydrogen sulphide through the solution rapidly until precipitation is complete and filter. Set aside 15 - 20 mls for titration after removal of the hydrogen sulphide (10 mins.) and buffer a 20 mls sample with 25% caustic soda to pH 6,0, measuring the amount used (2 - 3 mls). Add 1 ml enzyme juice and aerate in blood urea apparatus for 30 minutes. Stop the aeration and add 2,0 mls 50% m-phosphoric acid, centrifuge, and titrate an aliquot of the supernatant fluid.

The difference between the two titres, due allowance having been made for the dilutions involved, represents vitamin C.

Micro-method.

The procedure is exactly similar in principle, smaller quantities being used and a micro-diffusion burette being used for the titrations.

20 mls urine or filtrate are refluxed with 5 mls stannous chloride solution (or 0,20 ml B.D.H. reagent) for 90 minutes. Clear with 10 mls mercuric acetate solution and titrate 1 ml or 2 ml aliquots. Half quantities are used for the enzymatic oxidation and a 2 ml aliquot is titrated by means of the micro-burette.

Some analyses of scorbutic urines carried out by
the method outlined above.

Table IX.

Ascorbic acid is expressed in mg/100 mls urine and
the non-vitamin reducing material in urine is
expressed similarly as ascorbic acid.

		Total indophenol reducing power.	Non-vitamin reducing material.	Ascorbic acid.	
Ur ₁ ... scorbutic urine, before and after treatment.					
Ur ₁	3	1,36	1,07	0,29	
Ur ₁	5	1,14	0,86	0,39	
Ur ₁	10	1,80	0,73	1,07	
Ur ₁	12	7,50	0,70	6,80	
Ur ₁	15	15,95	0,70	15,25	
Ur ₁	16	6,70	0,79	5,91	
Ur ₁	17	16,30	1,15	15,15	
Ur ₂ ... scorbutic urine, before treatment.					
					Daily excretion.
Ur ₂	6	1,23	0,62	0,61	5,96 mg
Ur ₂	7	0,53	0,61	-----	-----
Ur ₂	8	2,50	0,73	1,77	16,6 mg
Ur ₂	9	2,27	1,28	0,99	7,1 mg
Ur ₂	14	1,04	0,72	0,32	5,7 mg
Ur ₂	16	0,87	0,63	0,24	4,2 mg
Ur ₂	17	0,83	0,59	0,24	4,0 mg
Ur ₂	19	0,71	0,62	0,09	1,3 mg
Ur ₂	20	0,86	0,61	0,25	2,9 mg
Ur ₃ ... scorbutic urine, before and after treatment.					
Ur ₃	11	0,75	0,65	0,10	1,4 mg
Ur ₃	14	1,01	0,68	0,33	4,7 mg
Ur ₃	18	0,50	0,53	-----	-----
Ur ₃	19	0,78	0,68	0,10	1,4 mg
Ur ₃	20	2,17	0,61	1,56	27,0 mg
Ur ₃	21	6,00	0,76	5,24	86,4 mg
Ur ₃	22	5,31	0,68	4,63	51,4 mg
Ur ₃	23	1,86	0,72	1,14	11,4 mg

Analyses of several dried vegetables used by

H.M. Navy.

The dried vegetables were extracted for a prolonged period with 5% m-phosphoric acid and the extract analysed. Due, probably, to the drying process, the vegetables were easily extracted, the solutions obtained being coloured but free from excess protein material.

Table X.

Vegetable.	mg ascorbic acid per 100 gms.
Julienne	2,6
French Beans	11,2
Turnips	10,8
Spinach	3,5
Cabbage	26,5
Onions	7,0

The values found are much the same as those quoted for cooked vegetables still retaining their tissue water but separated from the cooking water. The value for spinach is decidedly low an interesting fact in relation to the relatively high heavy metal content of spinach.

SUMMARY.

The method of Scarborough and Stewart (1937) for the determination of ascorbic acid in urine has been investigated and modified.

A new method using stannous chloride in dilute hydrochloric acid solution for the reduction and hydrolysis of urine followed by enzymatic oxidation to determine total vitamin C has been developed. The results of the two methods are in good agreement.

Ascorbic acid and perhaps other indophenol reducing substances are liberated during hydrolysis of urine from non-reducing complexes.

The specificity of the enzyme under the conditions of analysis was investigated. The rate of oxidation of substances other than vitamin C was not significantly greater in presence of the enzyme than in its absence.

Some analyses are reported. Urines from scorbutic subjects show very low concentrations and daily outputs of vitamin C and occasionally none is detectable.

Dried vegetables retain a fair amount of vitamin C and constitute a useful source of this vitamin.

Examination of the ascorbic acid content of various vegetable juices, the ascorbic acid content of vegetable juices was found to be very low. The ascorbic acid content of vegetable juices was found to be very low. The ascorbic acid content of vegetable juices was found to be very low.

PART II.

INTRODUCTION.

Ascorbic acid oxidase.

The oxidation of ascorbic acid by plant tissues was observed by Sz. Györgi (1928) who later postulated a specific enzyme responsible for this oxidation in vivo and possibly connected with the natural function of the vitamin. The same general phenomenon was observed by many other investigators: Zilva, Tauber and Kleiner, Srinivasan, Hopkins and Morgan, who used widely different plant tissues such as apple, hubbard squash, cucumber, cabbage, cauliflower, etc., and they concluded that the reaction was an enzymatic one. The dynamics of the reaction and its relationship to glutathione were worked out by Hopkins and Morgan (1936) who stimulated general interest in the oxidase. A certain amount of confusion has existed, however, since Barron et al., (1935) showed that ascorbic acid was not auto-oxidisable at pH values less than 7,6 and that the oxidation was due solely to minute traces of copper which acted as catalyst, and also that on examination of the amounts of copper in many vegetable juices, the oxidation of ascorbic acid by those juices was MAINLY due to the relatively large concentrations of copper ions in the juices.

Haemochromogens also act as catalysts for the reaction. Stotz, Harrer, and King (1937) further showed that the "natural" enzyme activity could be imitated by loose combinations of copper ions and protein and suggested that natural juices, which contain protein and copper ions, oxidised ascorbic acid by virtue of the mutual presence of these two factors and, consequently, there was no need to postulate the existence of a true ascorbic acid oxidase. It should be noted, however, that the amounts of copper present in the "synthetic enzymes" were greatly in excess of the amounts of copper in the ionic form required to catalyse the reaction at the same rate: in other words, protein, while imposing upon the copper ions the qualities of an enzyme such as heat lability and variation of activity with the hydrogen ion concentration, hindered their catalytic activity. Furthermore, the copper could be removed from the "synthetic enzyme" by simple dialysis against water but in the case of vegetable juices, only part of the copper was removable in this fashion. Dialysis against dilute acid removes all the copper from the natural juices and renders them inactive: it would seem, therefore, that in the case of the vegetable juices, the copper is bound much more firmly to the protein and is not fortuitiously combined as in the "synthetic enzymes".

While it is true that part of the oxidase activity of vegetable juices is due to the mutual presence of copper ions and protein as well as other catalysts such as haemochromogens, the evidence discussed above does not exclude the existence of an individual and discrete ascorbic acid oxidase; Spruyt and Vogelsgan (1938) concluded that the catalysis by ionic copper in the presence of protein was insufficient to explain the great oxidase activity of plant extracts.

More recently, Lovett-Janison and Nelson (1940) have isolated from Summer crook-neck squash an enzyme which contains 0.15% copper, is free from peroxidase activity, catalyses the oxidation of ascorbic acid and is dissimilar from polyphenol oxidase.

The experiments about to be described provide evidence from a different source for the existence of a discrete ascorbic acid oxidase and also show that the prosthetic group of this enzyme is copper.

Copper proteins.

A number of enzymes is now known in which the prosthetic group is a metal. The best known of these are copper proteins, iron proteins, manganese proteins and a zinc protein. The copper proteins whose identities have now been established are as follows:

Polyphenol oxidase
 Laccase
 Tyrosinase
 Haemocyanin
 Haemocuprein
 Hepatocuprein
 Ascorbic acid oxidase.

Kubowitz (1937, 1938) has isolated from potato an enzyme which oxidises polyphenols and is variously known as polyphenol oxidase and other names derived from the substrates. The enzyme in its purest state contains some 0,2% copper and is inhibited by cyanide, hydrogen sulphide and carbon monoxide. Prolonged dialysis against water does not affect the amount of copper in the enzyme or its activity but dialysis against dilute mineral acid or cyanide inactivates it completely by removing the copper. Kubowitz has succeeded in removing copper from both polyphenol oxidase and haemocyanin by this method and, in the case of the enzyme, has restored the activity by the addition of inorganic copper salts, so reconstituting the enzyme "molecule".

Laccase, haemocuprein, hepatocuprein and tyrosinase all have a copper content of approximately 0,2%, some of them being bluish-green or blue in the pure state.

Haemo- and hepato-cuprein were isolated from ox blood cells and liver respectively by

Keilin and Mann. Laccase (Keilin and Mann) and tyrosinase (Dalton and Nelson) are both enzymes of the polyphenol oxidase type.

Lovett-Janison and Nelson isolated ascorbic acid oxidase from summer crook-neck squash (*C. pepo condensa*), this source being superior to cucumber, cauliflower, apples, parsnips, carrots, spinach, string beans, cabbage, green peppers and oranges. The pure preparations contains 0.15% copper; traces of manganese bear no relation to the activity whatever and added manganese does not activate the enzyme, thus proving dissimilarity from dihydroxymaleic acid oxidase (Theorell, 1939). The pure preparations were almost entirely free from peroxidase activity and were inactive towards p-cresol and only very slightly active towards catechol and hydroquinone. The method of expressing activity used by these authors makes it very difficult to find a suitable basis for comparison with the oxidase from cucumber described here so that no opinion can be advanced about the possible identity of the two enzymes. Both, however, show great catalytic activity in the oxidation of ascorbic acid, both contain copper in amounts related to the activity and both fail to catalyse the oxidation of polyphenols to a significant extent.

Ferrocyanide (10 gms each of potassium ferrocyanide and barium hydroxide).

The distillate of slightly more than 9 litres

EXPERIMENTAL PROCEDURE.

The general technique used for the study of the oxidase-ascorbic acid system was the well-known Warburg manometric technique. The enzyme and other substances under consideration were placed in the main space of the reaction vessels with an air gas phase, 10% potassium hydroxide in the centre cup, and a known quantity of ascorbic acid, usually 25 micromolecules dissolved in 0.20 ml water in the side bulb. The reaction was started by tipping the ascorbic acid into the main space after temperature equilibration had been reached at 25°C. The manometers were filled with Brodie's fluid (sp.gr. 1.034) and the flasks were calibrated with their manometers both by filling with mercury and by the oxygen uptakes of weighed amounts of ascorbic acid.

The main difficulty was the preparation of large quantities of pure water for buffer solutions, dialysis, washing, etc., since impurities of copper ions would invalidate many of the experiments. The following procedure was found to be satisfactory. Water from the laboratory electric still was distilled in 10 litre amounts from an alkaline solution of potassium ferrocyanide (10 gms each of potassium ferrocyanide and barium hydroxide). The distillate of slightly more than 9 litres

was then distilled from a mixture of potassium permanganate and baryta (10 gms each) and the distillate transferred to an all-glass PYREX still incorporating a 12-bulb fractionating column where the water was distilled repeatedly until samples taken for the preparation of buffers and solutions showed a smaller uptake than 5 c.mm. per hour when shaken with ascorbic acid at pH 6,0. Three such distillations were usually necessary, the ultimate yield of pure water - i.e., copper-free water - being 5 litres. The water was stored in large PYREX bottles and is hereinafter referred to as distilled water. pH = 6,5.

Cleaning.

All apparatus was of PYREX glass and was cleaned before use by :-

- 1) boiling for 5 minutes in water with Lux soap flakes.
- 2) washing with tap water and distilled water.
- 3) immersion in cleaning fluid consisting of equal parts concentrated nitric and sulphuric acids.
- 4) washing with ordinary distilled water and distilled water before drying in an air oven.

I wish to stress the importance of the precautions taken to avoid contamination of the reagents with copper.

Buffer solutions.

The following three series were used :-

Acetate: Molar, pH 6,0
 McIlvaine's citro-phosphate, M/10, pH 6,0.
 Clarke's phosphate, M/4, pH 6,0.

The pH was tested in the Beckman pH meter and all samples were tested for the presence of traces of copper as in the case of distilled water.

Ascorbic acid solution.

A stock solution was prepared containing 0,1100 gm in 5,00 mls water, 0,20 of which contains 25 micromolecules, corresponding to an oxygen uptake of 280 c.mm. The solution was titrated from time to time against standard iodine and no variation was observed.

Dialysis.

This operation was carried out in cellophane sacks of unknown porosity. The ratio of the volume of cucumber juice to the volume of dialysate was kept constant after the with-drawal of samples, and the dialysate was changed every 24 hours. Dialysis proceeded at 0°C and every care was taken to prevent contamination of the fluids.

The ratio of the volume of fluid dialysing to the volume of dialysate was fixed at 1 : 4, so that 4 mls dialysate are equivalent to 1 ml cucumber juice. Thus, by preparing buffers and reagents from a quantity of the dialysate 4 mls dialysate will be used in the preparation of

the reagents required to fill one reaction vessel and any copper in the dialysate will catalyse the oxidation of the ascorbic acid tipped in. This rate of oxidation depends on the amount of copper in the 4 mls dialysate and hence corresponds to the copper dialysed from 1 ml cucumber juice.

Enzyme preparations.

The source of the enzyme was cucumber juice prepared by autolysing the cucumber. After various methods had been tried, it was found that the simplest and most effective method of freezing the tissue was to allow a whole cucumber to freeze overnight in the cold chamber of the ice chest. The frozen tissue was sliced into convenient sections of about $3/8$ inch thick and placed in a large filter funnel, the neck of which held a porcelain grid or a loose plug of cotton wool. On standing thus for some hours at room temperature, the cells autolysed and the juice was collected in a large PYREX flask under the funnel: the juice was stored in the ice chest. This method yielded a larger volume of clearer juice than any other method and avoided the necessity of treating the juice to remove any extraneous matter.

Warburg manometric method.

The reaction vessels were flasks of the single side-bulb type. The routine method

involved the following quantities of reagents :-

- 2,00 mls buffer solution, pH 6
- 0,20 mls ascorbic acid solution
- 0,20 mls 10% potash with filter paper roll
- 0,20 mls of a suitable concentration of other reagents such as CuSO_4

The total volume was made up to 4,20 mls with distilled water. The volume of reactants was 4 mls.

The levels in the manometers were read every 5 minutes after tipping except in the cases of extremely small oxygen uptakes when the interval of time was suitably increased. Control flasks containing 1) ascorbic acid in buffer and 2) no ascorbic acid were set up each time: the former served to check the purity of the reagents and the latter acted as a blank for the other reagents including the cucumber juice. A thermobarometer was also set up each time.

The tests carried out to detect impurities in the reagents and distilled water were carried out in acetate buffer, there being no slight inhibition as in the case of phosphate buffer. The test lasted for 2 hours and a flask containing the inhibitor sodium diethyldithiocarbamate was set up as an additional check.

Incinerations.

Analysis of the ash of cucumber juice was carried out by evaporating the juice in a platinum basin at 60 - 70°C before incinerating in a platinum crucible, great care being taken to prevent loss of material by spurting. "Blank"

incinerations were done each time before the experiment to detect any traces of copper both by a qualitative test with diethyldithiocarbamate and a test for catalytic oxidation of ascorbic acid. These tests proved negative provided that the crucible and basin had been cleaned by a sodium sulphate fusion.

Copper estimations.

Copper was determined colorimetrically by means of the sodium diethyldithiocarbamate reagent (0.1% solution) in matched 50 ml Nessler tubes. Attempts to estimate the extremely minute traces of copper found in the juice after dialysis by means of a photoelectric colorimeter or a photometer were fruitless since the yellow colour was too faint to be matched accurately and the extinctions were too small to be significant. By means of Nessler tubes, however, analyses could be carried out rapidly and accurately with ease. Rough determinations were done by preparing a series of standards from dilutions of copper sulphate with one of which the "unknown" was matched. More accurate determinations were carried out by making up the "unknown" tube and adding a very dilute solution of copper sulphate from a burette to the reagent in another Nessler tube until the colours matched: this procedure is valid for this colour reaction since

the quantity of copper in one Nessler tube - about 0.01 microgram - is so small that the colour complex formed may be considered to be in true solution.

It was also possible to calculate the amount of copper in a sample of ash from the rate at which it catalysed the oxidation of ascorbic acid.

The relation between the rate of oxidation and the total amount of copper catalysing the reaction was determined empirically and the data used to calculate the amounts of copper in the ash from enzyme preparations. These estimations were carried out under standard conditions using acetate buffer.

all oxidation except that of ascorbic acid. For example, catalytic oxidation of ascorbic acid, which possesses, relatively low catalytic activity, of the catalytic power of some metals, or excluded from the experiments. The results of enzyme preparations since the results of iron with the 0.01 microgram range of negative or so weakly positive effect of haemochromogen (assuming that the iron was present as such) would be insignificant small.

Some experiments were also carried out with copper sulphate as catalyst in amounts ranging from 0.0001 to 0.01 microgram. Each reaction varied about 10% of the control.

DISCUSSION and RESULTS.

Barron, Klemperer and De Meio (1935) showed that by rigid purification of reagents to avoid contamination with heavy metals, solutions of ascorbic acid in buffers were quite stable and did not oxidise in presence of air. (Below pH 7,6). They further showed that copper was the only metal among several examined which catalysed this atmospheric oxidation. These facts provide a suitable starting point for the examination of the enzymatic oxidation of ascorbic acid since complete control of oxidation can be accomplished by arranging the experimental conditions to exclude all oxidation except that by an enzyme. For example, catalytic oxidation by haemochromogens, which possess, molecule for molecule, a fraction of the catalytic power of ionic copper, is excluded from the experiments with dialysed enzyme preparations since qualitative tests for iron with the α -dipyridyl reagent were either negative or so weakly positive that the amount of haemochromogen (assuming that all the iron was present as such) would be insignificantly small.

Some experiments were first conducted with copper sulphate as catalyst in amounts ranging from 0,000001 mM to 0,001 mM in each reaction vessel along with 25 micromolecules

ascorbic acid at pH 6,0. The results, shown in Table XI, are in fair agreement with those published by Barron et al.

Table XI.

pH = 6,0. Temperature = 25°C.

Substrate, 25 micromoleculcd ascorbic acid.

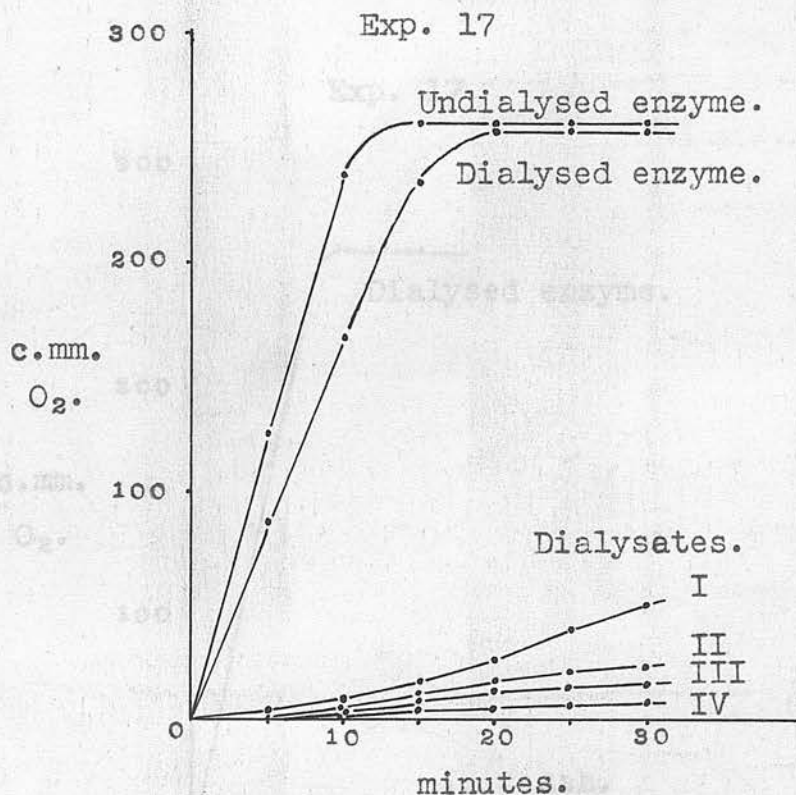
Copper sulphate mM.	Oxygen consumption c.mm. per hour.
0,000001	7
0,000005	39
0,00001	71
0,0001	221
0,001	709

Under the same conditions, 1,00 ml autolysed juice from cucumber produced an oxygen uptake of approximately 1500 c.mm. per hour.

In order to obtain solutions of the oxidase which would be free from adventitious heavy metals etc., the vegetable juice was dialysed for several days until the dialysate when tested for catalytic activity towards ascorbic acid showed none. The activity of the juice being dialysed decreased slightly from day to day during the period of dialysis of 6 - 7 days until the rate of oxidation catalysed by 1 ml of juice was about 1000 c.mm. per hour.

The following graph illustrates such an experiment

Figure 8.



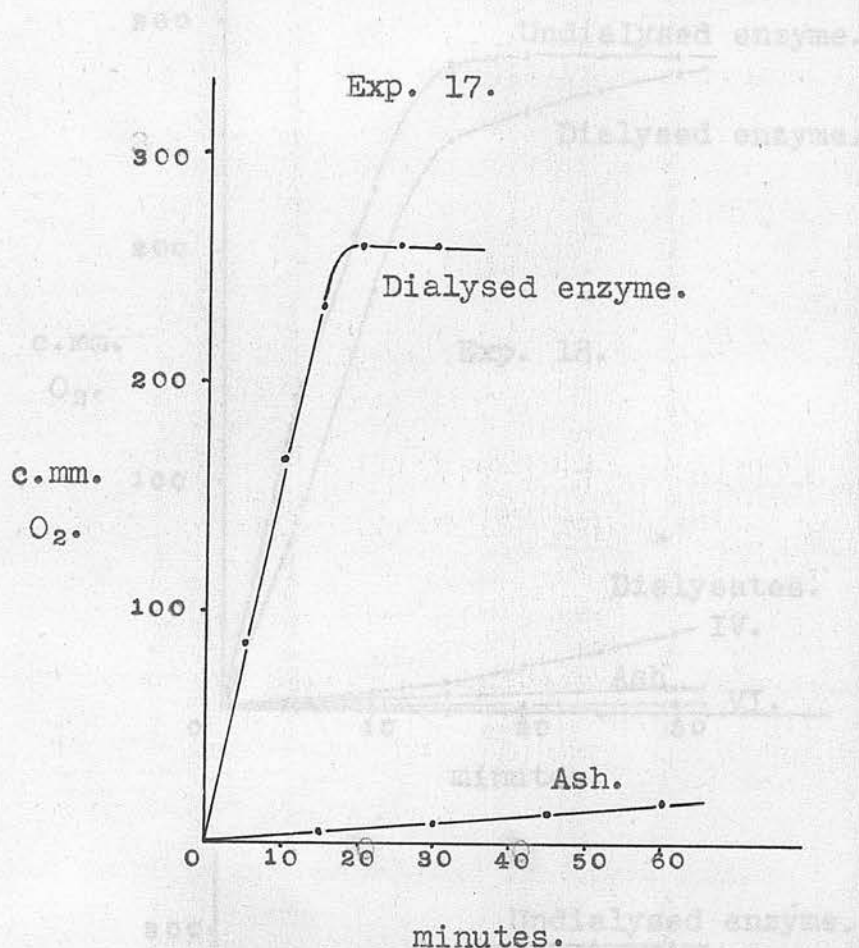
All inorganic matter having been removed along with quinonoid and phenolic materials and organic molecules of small dimensions from the enzyme preparation by dialysis, some 50 mls were measured out for incineration, the resulting ash taken up and tested for copper as previously described.

The results are shown below in Figure 9.

Figure 9.

Cu/ml found	Activity of ash c.mm. O_2 /hr.	Cu calculated from activity.
0,002	16	0,0022

Figure 9. (contd)



The activity of the dialysed enzyme preparation is, therefore, much greater than the activity of the same amount of copper in the ionic state in the ash. The curves above are for the catalytic activities of 1 ml enzyme preparation (dialysed) and 1 ml of a solution of the ash after the latter had been made up to the original volume of the enzyme preparation.

Three further experiments illustrating the same principle are recorded below in Figure 10.

Figure 10.

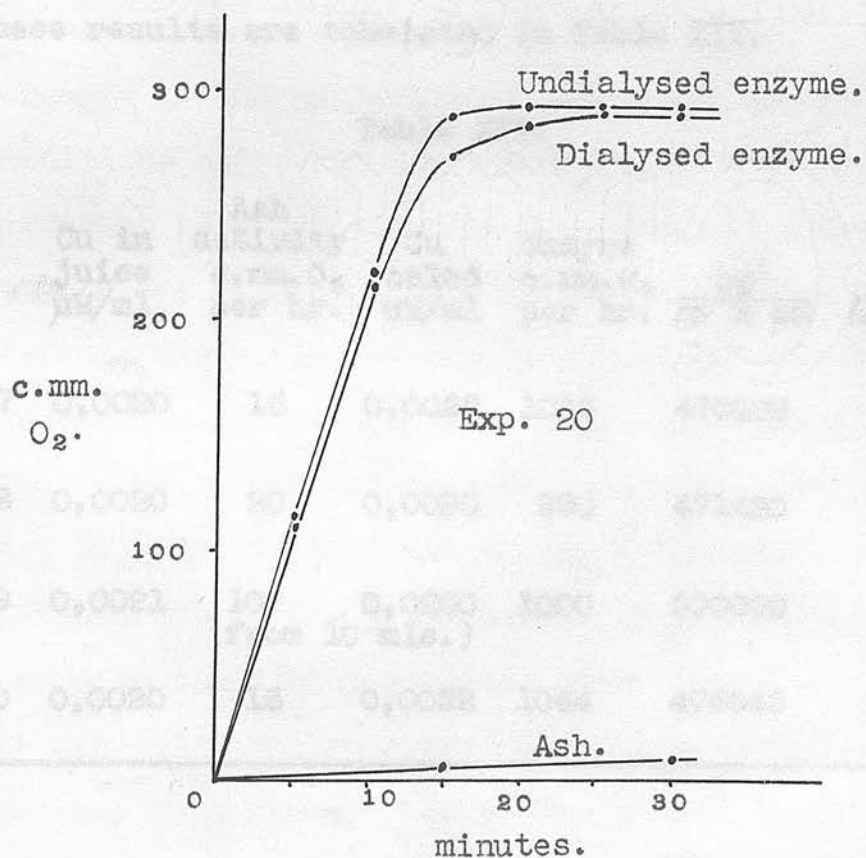
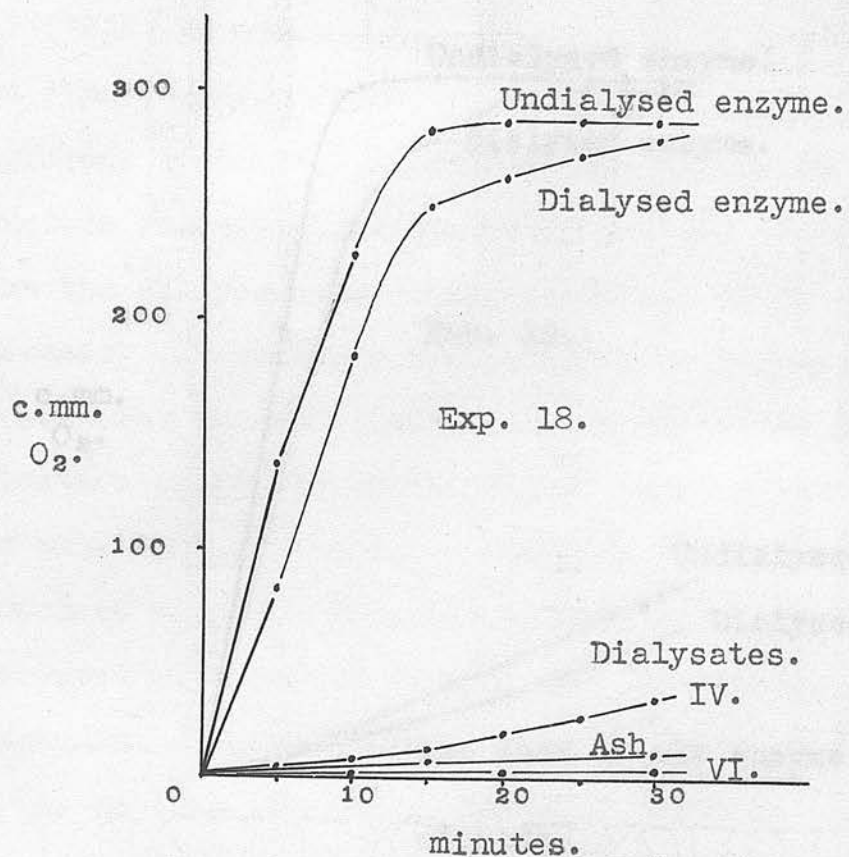
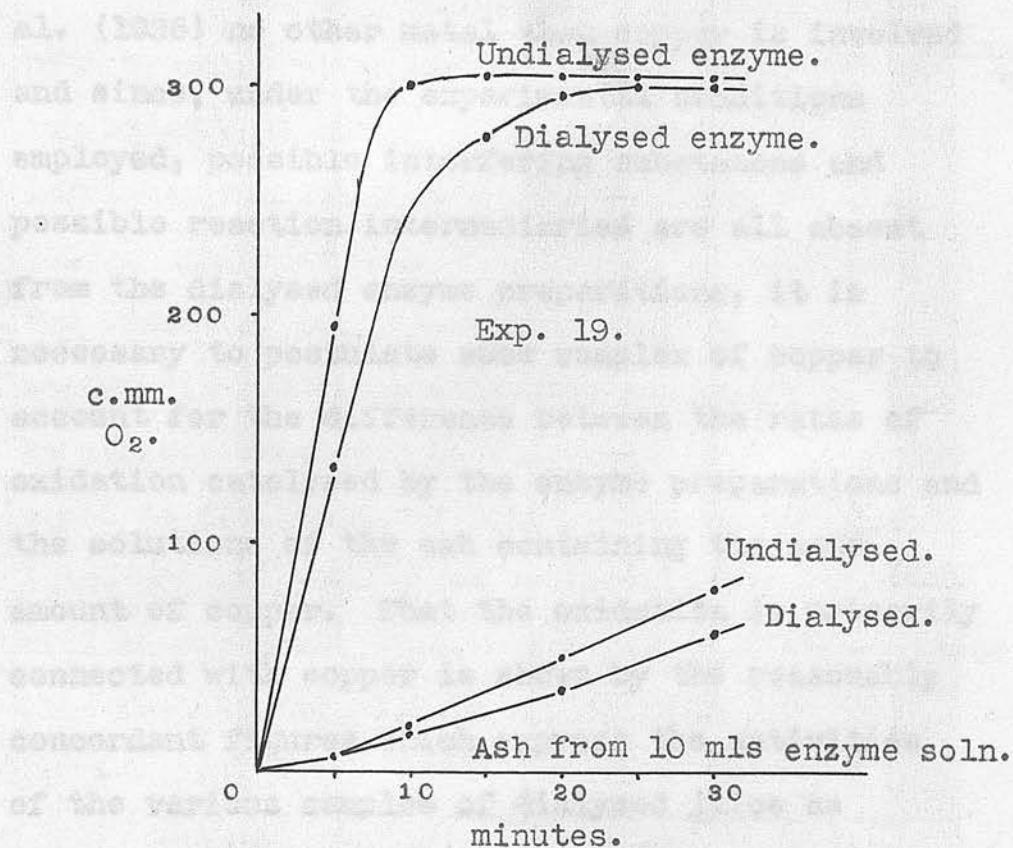


Figure 10. (contd).



These results are tabulated in Table XII.

Table XII.

	Cu in juice $\mu\text{M/ml}$	Ash activity c.mm.O ₂ per hr.	Cu calcd $\mu\text{M/ml}$	Enzyme c.mm.O ₂ per hr.	$\frac{\text{O}_2}{\text{hr} \times \mu\text{M}}$	$\frac{\text{O}_2}{\text{min} \times \gamma}$
Ex17	0,0020	16	0,0022	1036	470909	123
Ex18	0,0020	20	0,0030	990	471430	126
Ex19	0,0021	102 (from 10 mls.)	0,0200	1000	500000	131
Ex20	0,0020	16	0,0022	1044	474545	124

Since, according to the results of Barron et al. (1935) no other metal than copper is involved and since, under the experimental conditions employed, possible interfering substances and possible reaction intermediaries are all absent from the dialysed enzyme preparations, it is necessary to postulate some complex of copper to account for the difference between the rates of oxidation catalysed by the enzyme preparations and the solutions of the ash containing the same amount of copper. That the oxidation is primarily connected with copper is shown by the reasonably concordant figures which express the activities of the various samples of dialysed juice as rate of oxygen consumption per micromolecule and per microgram of copper. (Table XII). The behavior of the preparations under the experimental conditions above and other conditions such as different hydrogen ion concentration and different temperature suggests that the copper complex is one with protein analogous to the copper protein isolated by Kubowitz from potato. Experiments with p-cresol and catechol as substrate, however, were negative in that the oxygen consumption was of the order of 5 - 10 c.mm. per hour (little more than the blank) compared with 1000 c.mm. per hour with ascorbic acid as substrate under the same conditions. The enzyme in the dialysed juice is clearly not a polyphenol oxidase or a

tyrosinase.

The catalytic oxidation of ascorbic acid both by copper ions and cucumber oxidase can be inhibited by a number of substances which form insoluble complexes with copper. (King et al., 1937). This has been confirmed. In addition, the amount of inhibitor which in this case was sodium diethyldithiocarbamate, necessary to to inhibit catalysis by an amount of ionic copper equal to the amount of copper in 1 ml dialysed juice was not sufficient to inhibit the action of 1 ml dialysed juice. An experiment illustrating this fact is recorded in Table XIII.

Table XIII.

Figures represent oxygen consumption in c.mm. per hour.

	No inhibitor	0,0005 mM inhibitor	0,000005 mM inhibitor
0,000002 mM CuSO_4	14	0	4
1 ml enz. preparation	1044	0	500

The excess of protein no doubt has some protective action against the formation of an insoluble complex of copper and so prevents complete inhibition.

The juice, after this treatment, was quite inactive in catalysing the oxidation of ascorbic acid and the results, being quite negative,

Inactivation of cucumber oxidase by dialysis
against cyanide and dilute acid.

Stotz, Harrer and King (1937) reported that the addition of cyanide and other inhibitors inactivated cucumber oxidase and that after dialysis to remove the inhibitors, the activity was restored in every case except that in which cyanide had been added. In view of the experiments of Kubowitz (1938) it seemed that the inactivation observed by Stotz, Harrer and King might be due to the removal of copper from the copper protein.

Samples of cucumber juice which had been dialysed for six days against distilled water were treated with sodium cyanide to give a concentration of the latter in the juice of $M/100$ and other samples were treated similarly with hydrochloric acid. The two mixtures were dialysed separately against distilled water for several days until tests for chloride and cyanide in the dialysates were negative. The successive dialysates, however, when tested for copper with diethyldithiocarbamate, gave positive reactions during the first 2 - 3 days of dialysis: there was, therefore, a further removal of copper from the juice by cyanide or dilute acid after simple dialysis against water had ceased to do this. The juice, after this treatment, was quite inactive in catalysing the oxidation of ascorbic acid and the results, being quite negative,

are not reported in detail.

Reactivation of cucumber oxidase after treatment
with cyanide and dilute acid.

It remained to attempt to restore the activity of the dialysed juice after removal of copper by dialysis against cyanide and dilute mineral acid in the same manner as that used by Kubowitz to resynthesise polyphenol oxidase.

Samples of dialysed juice and of known copper content were dialysed against M/100 sodium cyanide and M/100 hydrochloric acid until all the cyanide and mineral acid had been removed (4 - 5 days). 3-fold and 10-fold amounts of copper were then added as copper sulphate solution and the mixture allowed to stand for a few hours at room temperature before a period of dialysis against distilled water for 24 hours to remove excess copper.

The preparations involving hydrochloric acid remained inactive after this treatment: this is possibly due to the extensive denaturation and precipitation of protein in the juice which occurred during the dialysis with dilute acid.

The preparations inactivated with sodium cyanide, however, were restored to activity by the addition of inorganic copper and the following table expresses the results of such an experiment.

The activity of enzyme Table XIV. has time

	Oxygen consumed c.mm. per hour.
A. 1 ml dialysed prep.	1248
B. 1 ml inactivated prep.	0
C. 1 ml inactivated prep. + 0,00002 mM CuSO_4 .	834
D. 1 ml inactivated prep. + 0,000006 mM CuSO_4 .	558

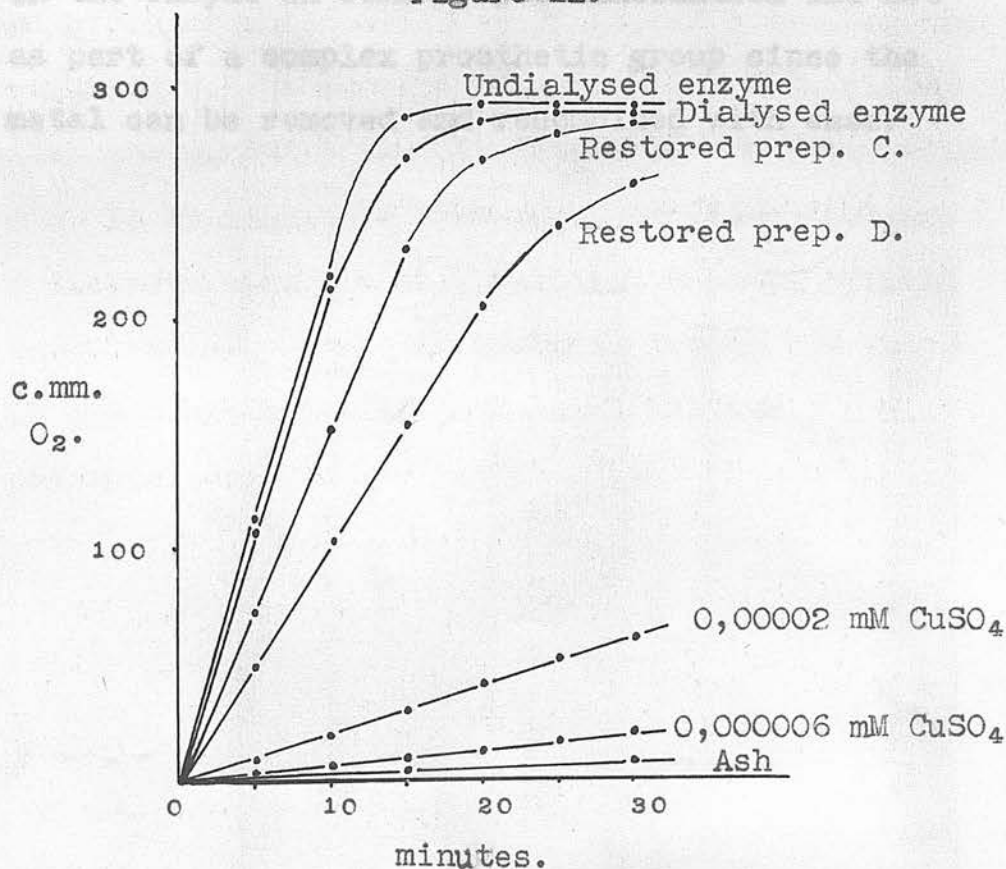
Cu content of dialysed prep. A. 0,0025 $\mu\text{M}/\text{ml}$.

Calculated from ash activity A. 0,0022 $\mu\text{M}/\text{ml}$.

Cu content of reactivated prep. C. 0,003 $\mu\text{M}/\text{ml}$.

The complete experiment is recorded graphically below, the curve for the oxygen uptake of the inactivated preparation being omitted as the uptake was nil.

Figure 11.



The activity of ascorbic acid oxidase has thus been successfully restored to the extent of 67% and 45% by the addition of 10-fold and 3-fold amounts of copper sulphate calculated from the copper content of the dialysed juice. The oxidase activity of dialysed cucumber juice and the activities of the restored preparations are greatly in excess of the catalytic activities of the Ash and the calculated amounts of copper alone. These facts, along with the inactivation of the enzyme by removal of copper and the relation between oxidase activity and copper content, provide evidence that ascorbic acid oxidase is a metal protein in which the prosthetic group is copper. It is probable that the copper is present in the enzyme in some simple combination and not as part of a complex prosthetic group since the metal can be removed and recombined with ease.

It is concluded that ascorbic acid oxidase is a discrete ascorbic acid oxidase, a metal protein in which the prosthetic group is copper and which is not identical with polyphenol oxidase preparations.

SUMMARY.

The oxidase activity of cucumber juice is proportional to the amount of copper in the juice after all the "free" copper and other extraneous materials have been removed by prolonged dialysis against pure water. This activity is much greater than the catalytic power of copper in the ionic state or copper in loose non-specific combination with protein.

Removal of the copper present in cucumber juice after long dialysis against water by further dialysis against cyanide or dilute mineral acid inactivates the oxidase.

Oxidase preparations inactivated by dialysis against cyanide are restored to activity by the addition of small excesses of copper sulphate, the restored enzyme showing greater catalytic activity than the amount of copper effecting the restoration.

It is concluded that cucumber juice contains a discrete ascorbic acid oxidase, a metal protein in which the prosthetic group is copper and which is not identical with polyphenol oxidase preparations.

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